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Review paper

What Morphology and Molecules Tell Us about the Evolution of Oligotrichea (Alveolata, Ciliophora)

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Abstract. The evolution of the dominant marine plankton ciliates, the oligotrichids and choreotrichids, is analysed for morphologic and genetic convergences and apomorphies based on literature and our own data. These findings have taxonomic implications. Within the oligotrichid genus *Parallelostrombidium* two subgenera, *Parallelostrombidium* Agatha, 2004 nov. stat. and *Asymptokinetum* nov. subgen., are established, using the courses of the ventral and girdle kineties as a distinguishing feature. Likewise, a different arrangement of extrusome attachment sites is used for a split of the oligotrichid genus *Novistrombidium* into the subgenera *Novistrombidium* Song and Bradbury, 1998 nov. stat. and *Propecingulum* nov. subgen.; *Novistrombidium* (*Propecingulum*) *ioanum* (Lynn and Gilron, 1993) nov. comb. and *Novistrombidium* (*Propecingulum*) *platum* (Song and Packroff, 1997) nov. comb. are affiliated. Based on discrepancies in the somatic ciliary pattern and the presence of conspicuous argyrophilic inclusions, the aloricate choreotrichid species *Pelagostrobilidium kimae* nov. spec. is distinguished from *P. conicum*. The diagnosis for the tintinnid family Eutintinnidae Bachy *et al*., 2012 is improved by including cell features. The co-operation of taxonomists and molecular biologists is strongly recommended to prevent misinterpretations of gene trees due to incorrectly identified species and for better species circumscriptions.

Key words: Choreotrichids, cladistic analyses, gene sequence analyses, oligotrichids, tintinnids, somatic ciliature.

INTRODUCTION

The marine plankton is a highly diverse community of organisms, among which we find the Oligotrichea, a ciliate taxon that episodically dominates the microzooplankton (Pierce and Turner 1992). The classification of the Oligotrichea in this paper follows Agatha (2004b) and is based on the relationships revealed by cladistic analyses and genetic phylogenies, except for the uncertain position of the halteriids (see below). As the Oligotrichea have species-specific trophic requirements (e.g., algivorous, bacterivorous, mixotrophic), proper identification is essential (i) for appreciating their role in the multi-step microbial food web and energy flux to the conventional planktonic food web and (ii) for estimating their biodiversity and biogeography (Agatha 2011a).

Since the last combined cladistic and phylogenetic analyses of the Oligotrichea, specifically of the tintin-

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nids (Agatha and Strüder-Kypke 2012a, b), there was a considerable progress, yielding a wealth of new genetic and morphologic data published in separate papers: (i) ~ 65 new SSU rRNA gene sequences (Bachy *et al.* 2012; Liu *et al.* 2012; Saccà *et al.* 2012; Santoferrara *et al.* 2013; Xu *et al.* 2012, 2013; Kim *et al.* 2013); (ii) cell features in two further genera (Saccà *et al.* 2012, Kim *et al.* 2013); and (iii) new somatic ciliary patterns in the tintinnid genus *Tintinnopsis* (Jiang *et al.* 2012) and the aloricate choreotrichid genus *Pelagostrobilidium* (Lee *et al.* 2011, Liu *et al.* 2012). The inclusion of these morphologic findings, new features (e.g., the somatic ciliary pattern evolution in *Pelagostrobilidium*), and ciliary patterns inferred from illustrations in Small and Lynn (1985) raised the number of considered taxa from 49 in Agatha and Strüder-Kypke (2012a, b) to 67 in the present review and the number of cladistically analysed characters from 76 to 94. Here, we thus present the current state of knowledge about the evolution of Oligotrichea based on concerted analyses of morphology and molecules.

Based on morphology and pattern of cell division, the Oligotrichea comprise the halteriids, oligotrichids, and choreotrichids (Agatha 2004b, Agatha and Foissner 2009). The most prominent feature of the Oligotrichea is the apical adoral zone of membranelles (C-shaped or circular arrangement of fan-like ciliary units), which is used for locomotion and filter feeding. The zone is divided into portions with large collar membranelles and small buccal membranelles. While the halteriids and oligotrichids contain exclusively aloricate species, the choreotrichids embrace besides naked species the tintinnids with loricae usually 50–300 µm long. Typically, the aloricate Oligotrichea have globular to obconical cell shapes, measure 15–260 µm in length, and have a reduced somatic ciliature with possibly sensory function. The tintinnid cells are attached by a peduncle to the bottom of the lorica, which they carry through the water. The cells are obconical in extended state and the anterior cell portion with the adoral zone of membranelles projects out of the lorica. Disturbance causes a retraction of the tintinnid into the lorica by contraction of the peduncle, and the cell assumes a globular to ellipsoidal shape. The somatic ciliature of tintinnids consists of numerous ciliary rows, which, in contrast to the aloricate taxa, are not exposed to the surrounding water, but are mostly covered by the lorica; it is assumed that the somatic ciliature is involved in lorica formation (Laval-Peuto 1981).

The first species assigned to the Oligotrichea were (i) the halteriid *Halteria grandinella* (Müller, 1773) Dujardin, 1841, (ii) the oligotrichid *Strombidium sulcatum* Claparède and Lachmann, 1859, (iii) the aloricate choreotrichid *Strobilidium caudatum* (Fromentel, 1876) Foissner, 1987, and (iv) the tintinnid *Tintinnus inquilinus* (Müller, 1776) Schrank, 1803. While the investigation of live and preserved specimens revealed only some characters in the aloricate taxa, the vase- or tube-shaped tintinnid loricae provided several features for identification and classification. In the 1950s, histological staining procedures commenced to reveal the ciliary patterns and allowed their usage in taxonomy and systematics. Notably, protargol impregnation, which stains basal bodies and the nuclear apparatus (macronuclei and micronuclei), is still routinely used today. The introduction of electron microscopy provided further insights into cell morphology, especially, into kinetid structure (basal body and associated root structures). A new era of phylogenetic analyses commenced with the introduction of gene sequencing. In particular, the small subunit ribosomal RNA (SSU rRNA) gene is frequently used to infer relationships among ciliate taxa.

The most recent monographs on halteriids, oligotrichids, and aloricate choreotrichids were based on live and preserved material only and regarded 127 species, 14 genera, and three families as valid (Maeda and Carey 1985, Maeda 1986). The application of silver-impregnation techniques has yielded many new species, whose number continuously increases. However, the rate of discovery was and still is distinctly influenced by the trend to neotypify species rather than to establish new ones, assuming that the majority of species have a cosmopolitan distribution. Accordingly, the intensity of taxonomic studies during the past thirty years was much higher than implied by the rate of discovery (Agatha 2011a). Currently, the number of reliable species amounts to 15 halteriid species in three genera and one family, 115 oligotrichid species in 18 genera and four families, and 50 species of aloricate choreotrichids in nine genera and five families; the majority of them have been redescribed, applying modern methods (live observation, silver impregnation, electron microscopy; own data). The more than one thousand tintinnid species are classified in 75 genera and 14 families, still mainly using lorica characteristics, as cell features have properly been described in only 29 species (Agatha and Strüder-Kypke 2012a, b; Jiang *et al.* 2012; Saccà *et al.* 2012; Kim *et al.* 2013).

The introduction of new investigation methods not only revealed new characters and contributed to the discovery of new species, but cladistic and phylogenetic analyses of these data also provided arguments for the establishment of new higher taxa and an improvement of the systematics (Agatha 2004a, b, 2011b; Agatha and Strüder-Kypke 2007, 2012a, b). Specifically, the evolution of the somatic kinetids and the somatic ciliary patterns turned out to be of high taxonomic and systematic significance within the Oligotrichea. The main clue for the evolutionary reconstruction was the orientation of the somatic kineties (ciliary rows), in particular the fact that only the anterior basal bodies of the dikinetids (basal body pairs) are associated with a cilium in the (dorsal) somatic kineties of the closely related euplotid (e.g., *Euplotes*) and hypotrich (e.g., *Oxytricha*) ciliates. In the following, the evolution of the oligotrichids and choreotrichids is reviewed, emphasizing homoplasies and apomorphies and their implications on taxonomy and systematics. The halteriids with their distribution mainly in freshwater and their controversial phylogenetic relationships (sistergroup to oligotrichids and choreotrichids according to morphology and mode of cell division, but members of the hypotrichs according to SSU rRNA analyses; Snoeyenbos-West *et al.* 2002, Agatha and Foissner 2009) are excluded here.

RESULTS AND DISCUSSION

1. Oligotrichids. The oligotrichids are the sistergroup to the choreotrichids. Both differ in the arrangement of the adoral zone of membranelles (C-shaped vs. circular pattern) and the origin of the oral primordium (oral apparatus of posterior division product develops in a subsurface tube vs. a pouch).

Although the somatic ciliature comprises only two kineties, namely, the ventral and girdle kineties with an ancestral kinetid structure, 13 patterns are currently known (Fig. 1). Four girdle kinety patterns occur in the tailed family Tontoniidae and also in tailless taxa (Figs 1II, IV–VII; Agatha 2011b). Due to the unique ultrastructure of the contractile tail (probably sea anchor function), Agatha (2004a) supposed an independent development of these four patterns in the tailed and tailless genera. This is actually supported by ontogenetic (positions of oral primordia) and genetic data (Figs 1IV, VI, 2). However, Agatha's hypothesis apparently failed concerning the monotypic tailless genus *Laboea* and the tailed genus *Spirotontonia* (Fig. 1VII), both with an identical somatic ciliary pattern. The genetic analyses by Gao *et al.* (2009) indicated that the pattern developed only once and that *Laboea strobila* lost the tail, regaining the plesiomorphic state; shared unique SSU rRNA regions, topology testing (Li *et al.* 2013), and some cladograms based on morphologic features support this assumption (Supplement Figs S3, S6, Tables S1, S2). The Tontoniidae apparently branched off rather early in the oligotrichid evolution (Figs 1, 2). The position of the oral primordium relative to the girdle kinety and extrusome attachment sites was a valuable taxonomic feature to split the speciose genus *Strombidium* and to establish two further genera, *Foissneridium* and *Opisthostrombidium* (Figs 1V, VIII, IX; Agatha 2011b).

The tailless genera *Apostrombidium* and *Varistrombidium* Xu, Warren, and Song, 2009 were established for strongly deviating, partially very complex somatic ciliary patterns (Xu *et al*. 2009). Agatha (2011b) supposed their origin in the tailless genus *Omegastrombidium* (Figs 1IV, XII, XIV); actually, the close relationship of the three genera is supported by ontogenetic and genetic data (Gao *et al.* 2009, Xu *et al.* 2011, Song *et al.* 2013; Fig. 2).

The tailless genus *Parallelostrombidium* is assumed to represent the most ancestral oligotrichid pattern (Fig. 1I). Differences in the extent to which the ventral kinety and the dextrally spiralled girdle kinety run parallel indicate the presence of two subgenera (see 'Taxonomic implications'; Figs S7, S8). The *Parallelostrombidium* pattern probably gave rise to the *Novistrombidium* pattern (Fig. 1II). Discrepancies mainly in the position of the extrusome attachment sites in relation to the oral primordium support two genetically distinct groups of *Novistrombidium* species (Figs 2, S10, S11; Li *et al.* 2013, Song *et al.* 2013), for which two subgenera are established (see 'Taxonomic implications').

In contrast to the great diversity of somatic ciliary patterns, whose evolutionary advantages are unknown, the oral ciliature is rather conserved in oligotrichids. Only in the genus *Cyrtostrombidium*, do the extraordinarily thick pharyngeal fibres and the absence of buccal membranelles and an endoral membrane justify the establishment of a distinct family, the Cyrtostrombidiidae (Agatha 2004a). A further family, the Pelagostrombidiidae, was established for freshwater genera characterized by a neoformation organelle (permanent tube, in which the posterior divider forms its oral apparatus; Agatha 2004a). The remaining genera are assigned to the family Strombidiidae, which is paraphyletic in cla-

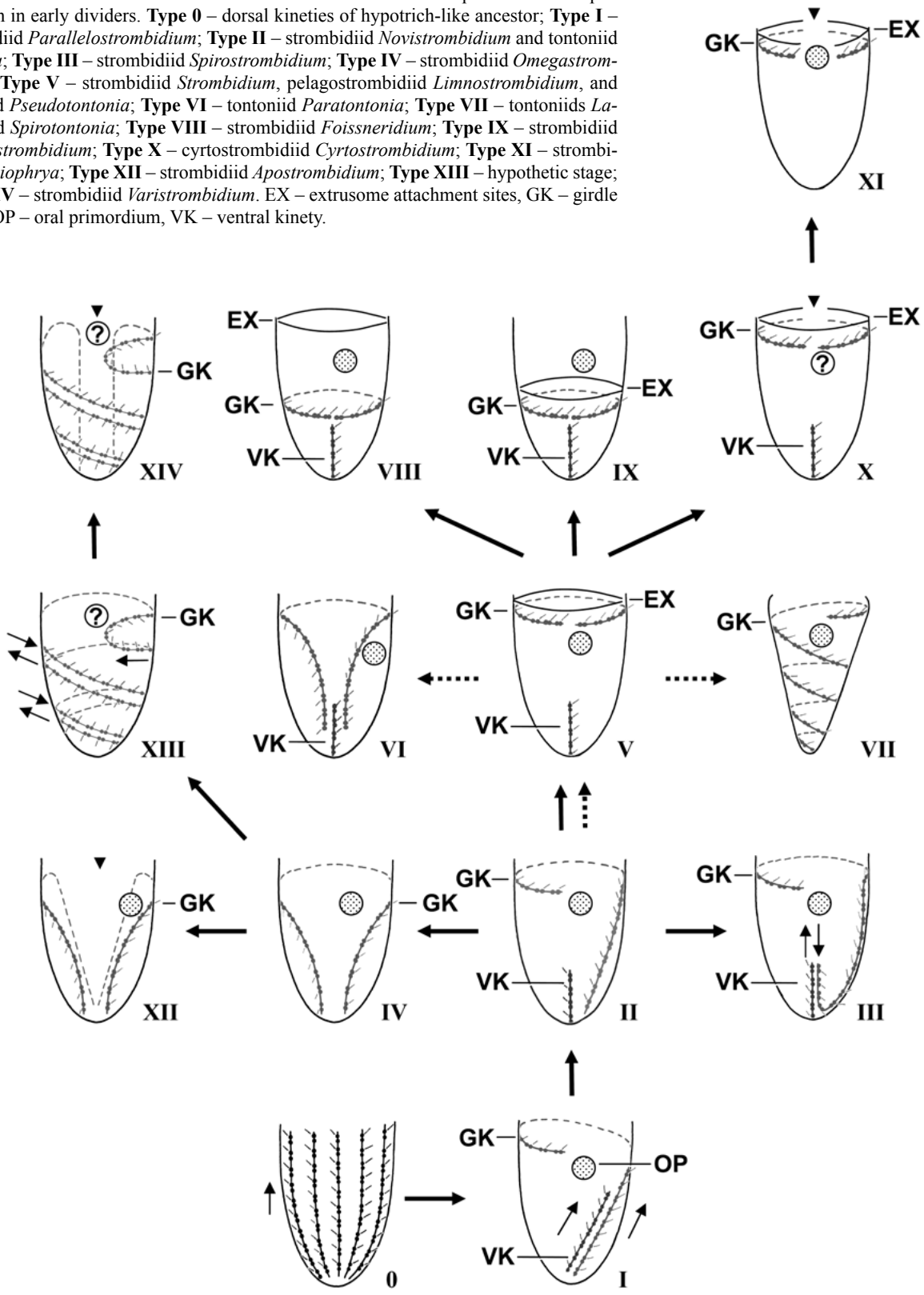

Fig. 1. Hypothetical evolution of oligotrichid somatic ciliary patterns (0–IV, VI, VII, after Agatha 2011b; V, VIII–XIV, originals; protargol impregnation). Small arrows mark orientation of kineties (posterior to anterior). Arrowheads denote dorsal breaks in girdle kinety. Dotted arrows mark the tontoniid evolution. Dotted circles denote position of oral primordium in early dividers. **Type 0** – dorsal kineties of hypotrich-like ancestor; **Type I** – strombidiid *Parallelostrombidium*; **Type II** – strombidiid *Novistrombidium* and tontoniid *Tontonia*; **Type III** – strombidiid *Spirostrombidium*; **Type IV** – strombidiid *Omegastrombidium*; **Type V** – strombidiid *Strombidium*, pelagostrombidiid *Limnostrombidium*, and tontoniid *Pseudotontonia*; **Type VI** – tontoniid *Paratontonia*; **Type VII** – tontoniids *Laboea* and *Spirotontonia*; **Type VIII** – strombidiid *Foissneridium*; **Type IX** – strombidiid *Opisthostrombidium*; **Type X** – cyrtostrombidiid *Cyrtostrombidium*; **Type XI** – strombidiid *Williophrya*; **Type XII** – strombidiid *Apostrombidium*; **Type XIII** – hypothetic stage; **Type XIV** – strombidiid *Varistrombidium*. EX – extrusome attachment sites, GK – girdle kinety, OP – oral primordium, VK – ventral kinety.

distic analyses (Figs S3–S6), as morphologic features for a split are currently not available; however, the arrangement of the extrusome attachment sites (clustered or in one or several rows) might be a promising feature, but more data are required for verification.

Gene trees consider only 11 out of the 18 oligotrichid genera and two out of the four families, and tree topologies vary, depending on the phylogenetic analysis used (compare unsupported nodes in Fig. 2 and supplemental Fig. S1). Nevertheless, several branches are supported by morphologic features (see above), especially by the evolution of the somatic ciliary patterns. While the family Tontoniidae and genera therein are fully supported in the phylogenetic analysis, support values for basal nodes in the family Strombidiidae are generally very low (Fig. 2).

2. Aloricate choreotrichids. Both cladistic and genetic analyses usually show (i) a monophyly of the tintinnids mainly based on the apomorphic lorica and (ii) a paraphyly of the aloricate choreotrichids (Figs 3, S2–S5, S12). Since different numbers of genera are considered in the cladograms and gene trees (aloricate choreotrichids: 9 vs. 6; tintinnids: 15 vs. 30), more detailed comparisons are difficult, especially since the tree topology is also influenced by the methods of analysis (compare Figs 3 and S2 for molecular analyses alone).

Immediately after separation from the oligotrichids, the structure of the somatic kinetids commenced to change in the choreotrichids, viz., a second cilium formed at the posterior dikinetidal basal body, producing a pattern found for instance in the aloricate genus *Strombidinopsis* (Fig. 4; Lynn *et al.* 1991). The subsequent steps occurred several times independently in (i) the tintinnids, (ii) the Lohmanniellidae and Strobilidiidae, and (iii) the genus *Lynnella*: the cilium at the anterior basal body is lost, and finally, the unciliated anterior basal body disappears. These comparatively rapid changes and the resulting diversity of kinetid structures in the choreotrichids contradict the structural conservatism of the somatic cortex hypothesized by Lynn (1981); but the reasons for and the advantages of these structural changes are unknown. In tintinnids, this transformation process is accompanied by the introduction of specialised ciliary fields and rows (see below).

The ventrally slightly opened adoral zone of membranelles in *Parastrombidinopsis*, *Parastrombidium*, and *Lynnella* is interpreted as synapomorphic retrogression to the plesiomorphic (open) state, as this feature is combined with an advanced kinetid structure, elongated bases (polykinetids) in the proximal collar membranelles, and a stomatogenesis within a pouch, features typical of choreotrichids (Fig. S12, Tables S1, S2; Agatha and Strüder-Kypke 2012a). In cladograms and gene trees, however, the position of *Lynnella* is highly variable, occasionally even suggesting an affiliation with the oligotrichids or a sistergroup relationship to the remaining choreotrichids (Figs 3, S2–S5, S12; Xu *et al.* 2012). The inclusion of gene sequences from *Leegaardiella*, *Lohmanniella*, and *Parastrombidium* will show whether (i) *Lynnella* is closely related to *Parastrombidium* and *Parastrombidinopsis* as indicated by the shape of the adoral zone and the latter two genera have to be affiliated with the family Lynnellidae or (ii) *Lynnella* is related to *Lohmanniella* as indicated by a similar structure of the somatic kinetids.

Usually, the genera *Pelagostrobilidium*, *Rimostrombidium*, and *Strobilidium* form a monophylum, the family Strobilidiidae, based on kineties composed of condensed monokinetids (single basal bodies) and cytoplasmic lips covering the bases of their cilia (Figs S3–S5, S12); this cluster is also revealed by molecular phylogenies (Figs 3, S2; Agatha and Strüder-Kypke 2007). The diversity of somatic ciliary patterns is comparatively large in *Pelagostrobilidium* (Figs S14–27). Probably, the *Rimostrombidium*-like ancestor had six more or less straight somatic kineties. The curvature of kinety 2 seems to be the most important feature for taxonomy and inferring intrageneric relationships: first, the kinety became anteriorly shortened, then sigmoidal, subsequently semicircular, and finally it performed a ~ 270° curvature. Posterior shortenings occurred several times independently in kineties 3–6. In the absence of kinety 5, kinety 6 performed distinct curvatures. Korean specimens identified by Lee *et al*. (2011) with *Pelagostrobilidium conicum*, as authoritatively redescribed by Agatha and Riedel-Lorjé (1998), deviate in the length of somatic kineties 1 (posteriorly shortened vs. unshortened) and 2 (anteriorly vs. posteriorly shortened) and an argyrophilic C-shaped structure near the collar membranelles (present vs. absent). These differences justify the establishment of a distinct species for the Korean specimens (see 'Taxonomic implications').

3. Tintinnids. The most conspicuous apomorphy of the tintinnids is the lorica, which probably acts as sea anchor (Jonsson *et al.* 2004) and may show a phenotypic plasticity caused by environmental conditions during its formation and the cell cycle (Laval-Peuto 1981, Agatha *et al*. 2012); the most reliable lorica fea-

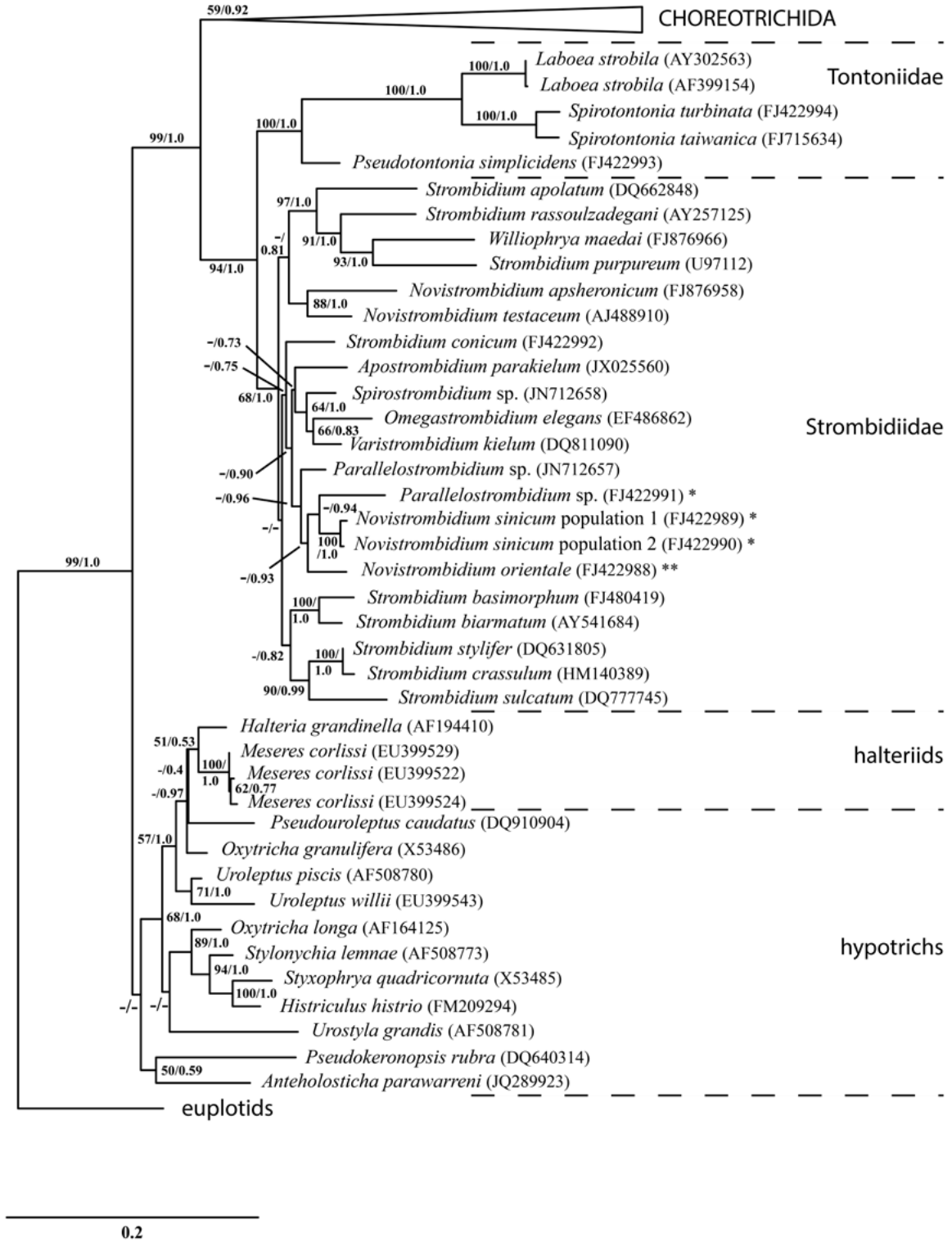

Fig. 2. Maximum Likelihood tree of the Oligotrichida inferred from small subunit ribosomal RNA (SSU rRNA) gene sequences (66 taxa and 1823 nucleotide positions) aligned with the Muscle algorithm (Edgar 2004) implemented in MEGA ver. 5.1 (Tamura *et al.* 2011). The alignment is available upon request. The tree was computed with RAxML (Stamatakis *et al.* 2008) and the datasets were bootstrap re-sampled 100 times. Support values are listed at the nodes. The second values at the nodes represent the posterior probability values of a Bayesian Inference analysis performed with MrBayes (Ronquist and Huelsenbeck 2003). Values below 50% and 0.5, respectively, are represented by a dash. * – initially published as *Spirostrombidium* sp.; ** – initially published as *Parallelostrombidium* sp.

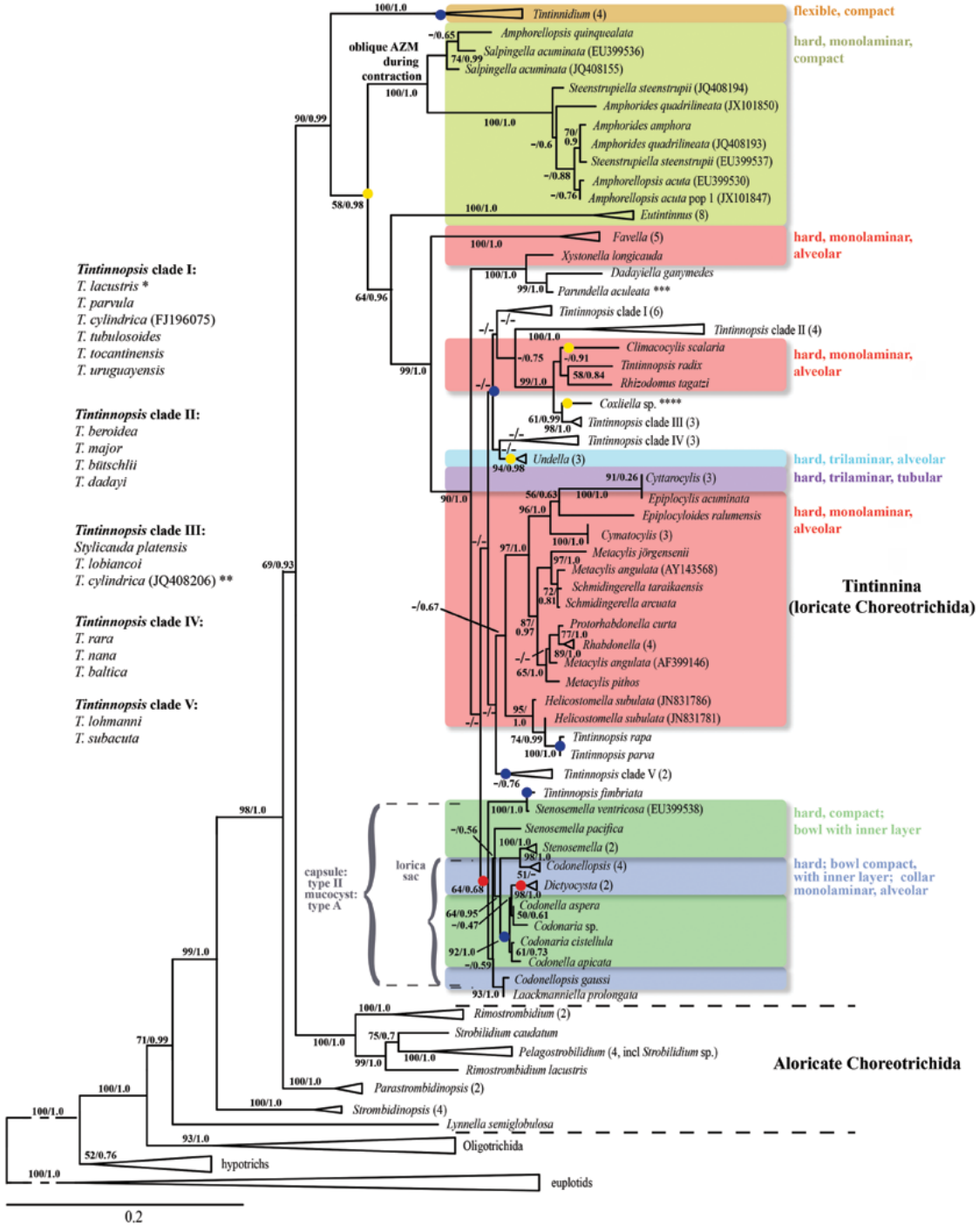

Fig. 3. Maximum Likelihood tree of the Choreotrichida inferred from small subunit ribosomal RNA (SSU rRNA) gene sequences (138 taxa and 1859 nucleotide positions) aligned with the Muscle algorithm (Edgar 2004) implemented in MEGA ver. 5.1 (Tamura *et al.* 2011). The alignment is available upon request. The tree was computed with RAxML (Stamatakis *et al.* 2008) and the datasets were bootstrap re-sampled 100 times. Support values are listed at the nodes. The second values at the nodes represent the posterior probability values of a Bayesian Inference analysis performed with MrBayes (Ronquist and Huelsenbeck 2003). Values below 50% and 0.5, respectively, are represented by dashes. Branches with unambiguously clustered taxa are collapsed, species of the genus *Tintinnopsis* grouped in 5 different clades numbered I–V. Most common lorica structures: ● – hyaline; ● – entirely agglomerated; ● – composed of hyaline collar and agglomerated bowl; * – after Kofoid and Campbell (1929) a synonym of *Codonella cratera*; ** – does not correspond with the redescription of Agatha and Riedel-Lorjé (2006); *** – possibly incorrectly identified, might be *Dadayiella acutiformis*; **** – invalid taxon, very likely a replacement lorica (see text).

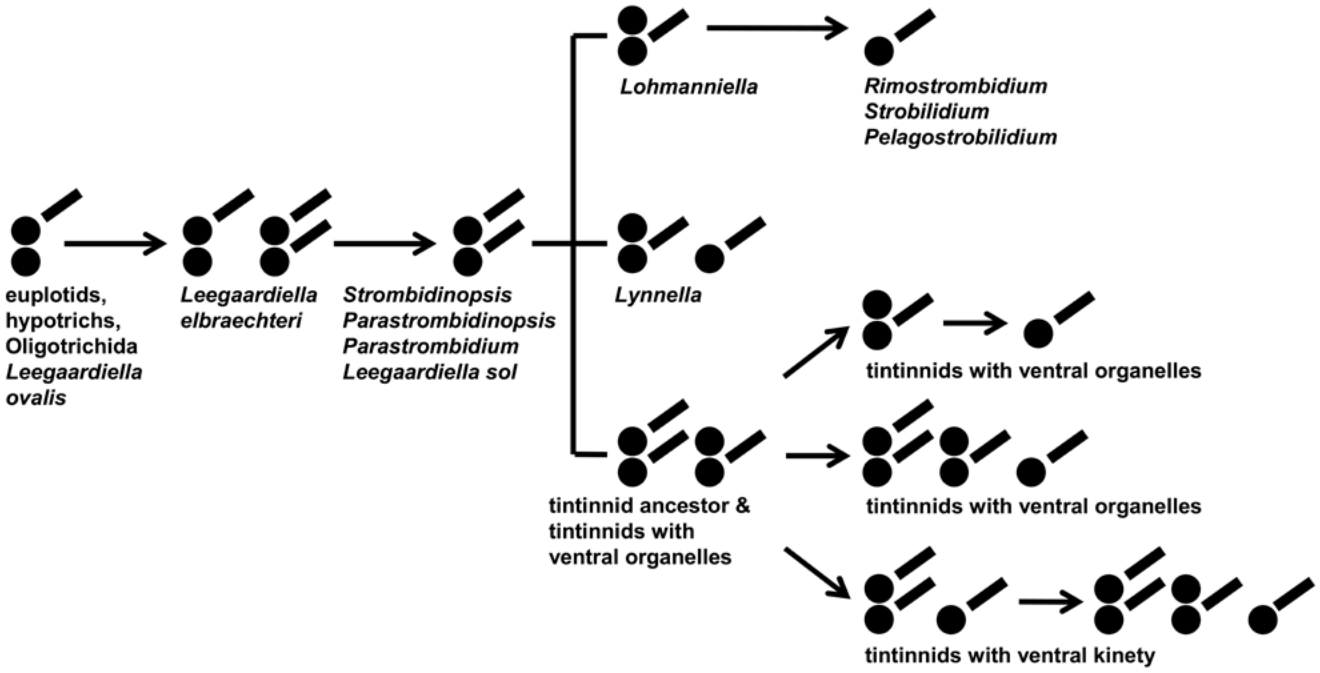

Fig. 4. Evolution of kinetid structures in the somatic ciliature of choreotrichid ciliates. The aloricate taxa have only one kinetid type, except for *Leegaardiella elbraechteri* and *Lynnella*. Tintinnids with ventral organelles have two (*Tintinnidium*, subgenus *Tintinnidium*), rarely one (*Tintinnopsis cylindrata*, *Membranicola*) or three (*Tintinnidium*, subgenus *Semitintinnidium*) kinetid types. Extant tintinnids with a ventral kinety have some dikinetids with two cilia and many monokinetids or some dikinetids with two cilia, some dikinetids with one cilium, and many monokinetids.

tures for species identification are apparently the general outline, details of the opening rim, the wall texture, and the opening diameter (Laval-Peuto and Brownlee 1986). Santoferrara *et al*. (2013) phylogenetically analysed variable regions of the SSU and LSU rRNA of tintinnids. They distinguished three taxonomic tendencies: (i) similarities and deviations in lorica shapes and sizes and gene sequences are consistent in the majority of morphospecies; (ii) some/several similar morphospecies show a high genetic divergence, supporting Kofoid's and Campbell's (1929, 1939) splits based on minute deviations in lorica morphology; and (iii) some/several morphologically distinct morphospecies are genetically identical or very similar. This has been shown for the genera *Cymatocylis*, *Favella*, *Rhabdonella*, and *Helicostomella*, in which distinct morphospecies deviate genetically by less than 1% in both the SSU and LSU rRNA sequences (Bachy *et al.* 2012, Xu *et al.* 2012, Kim *et al.* 2013). However, conspecificity cannot automatically be inferred from a high genetic similarity as demonstrated in two *Helicostomella* morphotypes. Despite a dissimilarity of only 0.5% in the SSU rRNA, compensatory base changes in the ITS2 helices II and III indicate the presence of two distinct species (Xu *et al*. 2012). Accordingly, the percentage of synonyms among the more than one thousand tintinnid species is still hardly guessable.

The genetic diversity within single morphotypes found by Kazama *et al*. (2012) markedly exceeds that of any previous molecular study on tintinnids, aloricate choreotrichids, and oligotrichids (see also Gong *et al.* 2013). For instance, the same *Tintinnopsis* morphospecies (with entirely agglomerated lorica) atypically clusters at the beginning of the tintinnid evolution with *Amphorellopsis*, *Steenstrupiella*, and *Salpingella* (all with hyaline loricae, a comparatively simple somatic ciliary pattern, and an oblique adoral zone in contracted cells; Figs S52–61) and also occurs in the tintinnid branch with the most complex ciliary pattern. Since previous and the present studies found the first occurrence of *Tintinnopsis* species only after the branching of *Favella* (Figs 3, S2, S3, S5, S12), methodological errors (misidentification of the species or contamination of the extracted DNA) might have caused these distinctly de-

viating sequences in single morphotypes. Accordingly, the sequences deposited by Kazama *et al.* (2012) in GenBank are not suitable as references in phylogenetic analyses.

Helical structures may constitute collars (epiloricae) or entire loricae of hyaline and agglomerated types. The *Coxliella* species with their completely helical loricae probably represent replacement loricae (paraloricae) built independently from cell division when lorica forming material is less abundant in the cell and construction is thus slower, or they are formed after cell division when the material is possibly less viscous (Laval-Peuto 1981, 1994). The presence of "*coxliella*" forms could be shown for *Favella* (Laval-Peuto 1981, Kim *et al.* 2010) and *Schmidingerella* (Agatha and Strüder-Kypke 2012a), whereas clear evidence from cultures is lacking for *Helicostomella*, *Parafavella* (all hyaline), and some *Tintinnopsis* species (entirely agglomerated; Laval-Peuto and Brownlee 1986). As already assumed by Brandt (1907), the genus *Coxliella* thus seems to be artificial, whereas Xu *et al.* (2013) regarded high abundances of *Coxliella* sp. and the apparent absence of a typical form as support for the validity of the genus *Coxliella*. The cell cycle of *Favella*, however, demonstrated that under certain (unknown) conditions the "*coxliella*" form can produce the spiralled "*decipiens*" form after cell division (Laval-Peuto 1981); so, the typical form must not necessarily occur. Further, the typical form might have been too rare to be detected in the study of Xu *et al.* (2013). In several other genera, helical structures seem to be absent, e.g., *Petalotricha*, *Salpingella* (both hyaline), *Codonella* (agglomerated), and *Dictyocysta* (partially or entirely hyaline; Agatha *et al.* 2012).

Both genetic and cladistic analyses reveal that different lorica types (hyaline, entirely or partially agglomerated) do not form distinct evolutionary lineages (Figs 3, S2–5, S12; Strüder-Kypke and Lynn 2008); only the branch of freshwater tintinnids with two ventral (ciliary) organelles is characterized by a special type, namely, a flexible and agglomerated lorica with a compact matrix. In the mainly marine tintinnids with a ventral kinety, the lorica walls were probably first hard, hyaline, and compact (*Amphorellopsis*, *Amphorides*, *Eutintinnus*, *Salpingella*, *Steenstrupiella*; the collapsible lorica of *Nolaclusilis* represents an autapomorphy), became hard, hyaline, and monolaminar with alveoli (Figs S28–38), and then the first hard, agglomerated loricae occurred, as indicated by the increasing complexity of the somatic ciliary pattern. Further hard, hyaline loricae are found in *Undella* (trilaminar with alveoli), *Cyttarocylis*, and *Petalotricha* (trilaminar with tubules; genetic data even indicate a synonymy; Bachy *et al.* 2012). Finally, the wall of the hard, agglomerated lorica portions became apparently bilayered, viz., composed of a thick outer layer with particles embedded into compact material and a continuous compact inner layer (Figs S39–51; *Codonella*, *Codonellopsis*, *Stenosemella*, and probably *Dictyocysta* and *Codonaria*). Hyaline collars that might be associated with such agglomerated bowls are compact (*Stenosemella*; own data) or alveolar (*Codonellopsis*, *Dictyocysta*; own data, Laval-Peuto 1994); however, the texture of the entirely hyaline and fenestrated lorica in *Dictyocysta mitra* seems to be tubular (Agatha 2010).

Together with the lorica, a right and left ciliary field evolved in the common tintinnid ancestor, giving rise to the mainly freshwater species with two ventral organelles and the mainly marine species with a ventral kinety. In the former branch, the diversity of kinetid structures is comparatively large, ranging from one to three types in a single species, while the complexity of the ciliary pattern did not change anymore (Figs 4, S12). In the marine branch, two kinetid structures first occurred together with the ventral kinety and were later completed by a third type, while the complexity of the pattern distinctly increased by the successive addition of a further field and specialised kineties (Agatha and Strüder-Kypke 2012b, this study).

Although the somatic ciliature is assumed to be involved in lorica formation, correlations between ciliary patterns, lorica types (hyaline, agglomerated), wall textures (compact, alveolar etc.), and deformability are not recognisable, except for the occurrence of a ventral kinety and hard loricae and ventral organelles and flexible loricae, respectively (Figs 3, S3–5, S12; Agatha *et al.* 2012). It seems more likely that differences in lorica type, texture, and deformability result essentially from differences in the lorica forming material, whose main chemical component is probably of proteinaceous nature with variable additions of, e.g., lipids and carbohydrates. However, a correlation between lorica features and the characteristics of the lorica material was again not evident (Agatha and Simon 2012, Agatha *et al.* 2012).

A detailed comparison of gene trees with cladograms is currently impeded by differences in the species and genera analysed (Figs 3, S3–5, S12). Nevertheless, the evolution of somatic ciliary patterns is the main feature complex in cladistic analyses of tintinnids and is rather well reflected by genetic phylogenies.

Further morphologic characters support several groupings in the molecular genealogies. The lorica sac with its foldable closing apparatus represents a synapomorphy of the genera *Codonaria*, *Codonella*, *Codonellopsis*, and *Dictyocysta* (Agatha 2010). *Stenosemella* and *Laackmanniella* are closely related to these four genera phylogenetically, but apparently lack a lorica sac (Fig. 3; Kim *et al.* 2013). While the relationship of the former genus might be explained by the same type of capsule (tintinnid extrusome; Laval-Peuto and Barria de Cao 1987), further morphological studies are required in the latter genus to understand its phylogenetic placement (Kim *et al.* 2013). An oblique orientation of the oral ciliature and peristomial field in contracted specimens probably represents the synapomorphy of *Salpingella*, *Amphorellopsis*, *Amphorides*, *Steenstrupiella*, *Salpingacantha*, and *Bursaopsis obliqua* (Figs S3, S12, S52–61). Actually, the former four genera constitute a monophylum in the gene trees separate from the genus *Eutintinnus*, for which Bachy *et al*. (2012) established the family Eutintinnidae; here, the family diagnosis is emended by including cell features (see 'Taxonomic implications').

The non-monophylies of tintinnid genera in gene trees necessitate further morphologic studies, especially, of the cells. Recently, the problem of two genetically distinct groups of *Favella* species was resolved and the genus *Schmidingerella* has been established for the second cluster deviating in ciliary pattern and lorica wall texture (Figs S28, S31; Agatha and Strüder-Kypke 2012a). A monolaminar, alveolar wall with surface ridges and pores unites the new genus, *Rhabdonella* (own data), *Rhabdonellopsis* (Gold and Morales 1977), *Protorhabdonella* (Kofoid and Campbell 1929), *Epiplocylis* (Abboud-Abi Saab 2008), and *Epiplocyloides* (Laval-Peuto 1994, Abboud-Abi Saab 2008) within the family Rhabdonellidae, which is mostly supported by genetic data (Figs 3, S2, S31–36). *Cymatocylis* also falls into this genetic cluster and indeed shows also ridges on the lorica surface (Laackmann 1910, Kim *et al.* 2013). A further conspicuous non-monophyly concerns the genus *Tintinnopsis*. Although as yet four different ciliary patterns have been discovered (Fig. S12), a reasonable split of the genus can, however, currently not be performed, as the cell features of its type species, *T. beroidea*, are unknown and the determination of the specimens sequenced is uncertain (Agatha and Strüder-Kypke 2012b).

Generally, doubtful identifications or misidentifications of genetically analysed species cause serious problems in the interpretation of the trees, particularly, when it concerns type species (see also above for Kazama *et al*. 2012). For instance, a very high genetic similarity (one base pair difference in the SSU rRNA) between *Dadayiella ganymedes* sequenced by Xu *et al.* (2013) and *Parundella aculeata* analysed by Bachy *et al.* (2012) was used as argument by Xu *et al.* (2013) to transfer the type species *D. ganymedes* to the genus *Parundella* and to make the genus *Dadayiella* thus invalid. The astonishing close genetic relationship of species affiliated with different families in the lorica-based classification (Tintinnidae and Xystonellidae) caused scepticism about the determinations. Actually, the micrograph and the morphometric data of the loricae indicate that the former specimens have been confused with *Dadayiella bulbosa* (with knob at posterior lorica process vs. without in *D. ganymedes*; Entz 1884; Brandt 1906, 1907). In the second case, the specimen genetically analysed perfectly matches *P. aculeata* in lorica shape and size (lorica length: 105 µm; opening diameter: 27 µm), but also *Dadayiella acutiformis* Kofoid and Campbell, 1939 (lorica length: 82–103 µm; opening diameter: 25–30 µm; Jörgensen 1924, Kofoid and Campbell 1939). The main differences between the two species are delicate longitudinal ribs in the anterior quarter of the *Dadayiella* lorica, which might be hardly recognisable in the light microscope when the cell is inside the lorica. The small micrograph provided by Bachy *et al.* (2012) does not allow any decision about the presence of ribs, and we assume that the authors have not seen the ribs and thus identified the specimen with *P. aculeata*. But nevertheless, the astonishing genetic relationship together with the existence of a similar-sized and similar-shaped *Dadayiella* species should keep us sceptical. If the latter specimen has actually been misidentified, both morphotypes would belong to the same genus (*Dadayiella*) and possibly even to the same species, as already suggested by Jörgensen (1924). However, any nomenclatural act should await a detailed re-investigation of the lorica and cell and possibly the analysis of additional molecular markers. These examples demonstrate the urgent need for co-operation of molecular biologists with experienced taxonomists for reliable identifications.

The consultation of monographs (e.g., Kofoid and Campbell 1929, 1939) is a very helpful first step, but species determinations should finally be based on original descriptions or authoritative redescriptions, as revising authors may have changed the circumscrip-

tions by lumping species. Synonymisations should be performed only after detailed investigations of the cell (live observation, protargol impregnation) and lorica ultrastructure (electron microscopy) and genetic analyses. The variable regions (D1-D2) of the LSU rRNA gene (Santoferrara *et al*. 2013) and ITS2 sequence and secondary structure comparisons (Snoeyenbos-West *et al.* 2003, Weisse *et al.* 2006) are promising molecular markers for elucidating phylogenetic relationships and species limits in tintinnids. Therefore, we regard any taxonomic acts merely based on gene sequence data and lorica features as premature, especially, as they are occasionally in conflict with the International Code of Zoological Nomenclature (ICZN 1999). On the other hand, descriptions of new species or redescriptions should comprise gene sequence analysis besides the complete morphologic data.

4. Taxonomic implications

Genus *Parallelostrombidium* Agatha, 2004

Subgenus *Parallelostrombidium* Agatha, 2004 nov. stat. (Fig. S7)

Diagnosis: Ventral kinety entirely parallel to girdle kinety.

Type species: *Strombidium rhyticollare* Corliss and Snyder, 1986.

Species assignable: *Parallelostrombidium* (*Parallelostrombidium*) *rhyticollare* (Corliss and Snyder, 1986) Agatha, 2004 and *Parallelostrombidium* (*Parallelostrombidium*) *siculum* (Montagnes and Taylor, 1994) Agatha, 2004.

Subgenus *Asymptokinetum* nov. subgen. (Fig. S8)

Diagnosis: Only posterior portion of erected ventral kinety parallel to girdle kinety.

Type species: *Parallelostrombidium paralatum* Xu *et al*., 2006.

Etymology: Composite of the Greek adjective *asymptotos* (not falling together) and verb *kinein* (to move), referring to the course of the girdle kinety, which continuously approaches the longitudinal ventral kinety; neuter gender.

Species assignable: The subgenus *Asymptokinetum* is monotypic, comprising only *Parallelostrombidium* (*Asymptokinetum*) *paralatum* Xu *et al*., 2006.

Genus *Novistrombidium* Song and Bradbury, 1998

Remarks: According to the topology tests by Li *et al.* (2013), the monophyly of the genus *Novistrombidium* cannot be rejected, which matches the Hennigian argumentation scheme (Fig. S6). Thus, the genus is split here only into two subgenera differing in morphology and probably in their ITS2 secondary structure (Li *et al.* 2013).

Subgenus *Novistrombidium* Song and Bradbury, 1998 nov. stat. (Fig. S11)

Diagnosis: Extrusome attachment sites in question mark-shaped pattern directly posterior to adoral membranelles and in an arc on posterior dorsal side. Oral primordium between question mark-shaped pattern of extrusome attachment sites and girdle kinety.

Type species: *Strombidium testaceum* Anigstein, 1913.

Species assignable: *Novistrombidium* (*Novistrombidium*) *testaceum* (Anigstein, 1913) Song and Bradbury, 1998 and *Novistrombidium* (*Novistrombidium*) *apsheronicum* (Alekperov and Asadullayeva, 1997) Agatha, 2003.

Subgenus *Propecingulum* nov. subgen. (Fig. S10)

Diagnosis: Extrusome attachment sites directly anterior to girdle kinety. Anterior portion of girdle kinety elongated, performing further dextral spirals. Oral primordium anterior to stripe of extrusome attachment sites extending along girdle kinety.

Type species: *Novistrombidium sinicum* Liu *et al*., 2009.

Etymology: Composite of the Latin prefix *prope* (near) and the noun *cingulum* (girdle), referring to the extrusome stripe directly anterior to the girdle kinety; neuter gender.

Discussion: *Novistrombidium* (*Propecingulum*) *sinicum* Liu *et al*., 2009 and *Novistrombidium* (*Propecingulum*) *orientale* Liu *et al*., 2009 are assigned to the new subgenus. While the affiliations of *Strombidium ioanum* and *S. platum* with the genus *Novistrombidium* are not disputable because of their dextrally spiralled girdle kinety abutting on a longitudinal ventral kinety (the orientation of the dikinetids indicates the presence of both a girdle and a ventral kinety in *S. platum*), their assignment to the subgenus *Propecingulum* has to be verified by live observations. However, the reticular silverline system directly anterior to the dextrally spiralled girdle kinety probably indicates the arrangement of the extrusomes in *S. ioanum*, which is therefore tentatively affiliated, becoming *Novistrombidium* (*Propecingulum*) *ioanum* (Lynn and Gilron, 1993) nov.

comb. Likewise, extrusomes were merely found in the posterior cell portion close to the girdle kinety in *S. platum*, which is thus also tentatively affiliated, becoming *Novistrombidium* (*Propecingulum*) *platum* (Song and Packroff, 1997) nov. comb.

Pelagostrobilidium kimae nov. spec. (Fig. S19)

Diagnosis: Size after protargol impregnation ~ 30 × 18 μm; obovoidal to pyriform. Invariably one micronucleus. Invariably six somatic kineties commencing at same level, except for anteriorly shortened kinety 2: longitudinal kineties 1 and 3–6 posteriorly shortened; kinety 2 slightly sigmoidal, on the left of kinety 3. About 24 collar membranelles and invariably one buccal membranelle.

Etymology: Dedicated to Y.-O. Kim (Korea Institute of Ocean Science & Technology, Geoje, Republic of Korea) due to her contributions to the ecology and taxonomy of marine planktonic ciliates.

Comparison with congeners: There is only one congener sharing the posteriorly shortened somatic kineties 3–6, namely, *P. conicum* as authoritatively described by Agatha and Riedel-Lorjé (1998). However, the Korean specimens deviate in the length of somatic kineties 1 (posteriorly shortened vs. unshortened) and 2 (anteriorly vs. posteriorly shortened) and an argyrophilic C-shaped structure near the collar membranelles (present vs. absent), justifying the establishment of a new species.

Family Eutintinnidae Bachy *et al.*, 2012

Improved diagnosis: Lorica cylindroidal with anterior and posterior openings at truncate ends, wall hyaline, rarely agglomerated, compact, with regular transverse striation in transmission electron micrographs. Usually four macronucleus nodules and two micronuclei. Somatic ciliature comprises (i) a right and left ciliary field with monokinetidal kineties having one dikinetid anteriorly, (ii) a short, monokinetidal ventral kinety, and (iii) two, rarely three dorsal kineties.

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Supplementary material: Figures S1–S61 and Tables S1–S3.

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